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(54) Title: ANTISENSE MODULATION OF MUCIN 1, TRANSMEMBRANE EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of mucin 1, transmembrane. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding mucin 1, transmembrane. Methods of using these compounds for modulation of mucin 1, transmembrane expression and for treatment of diseases associated with expression of mucin 1, transmembrane are provided.



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ANTISENSE MODULATION OF MUCIN 1, TRANSMEMBRANE EXPRESSION**FIELD OF THE INVENTION**

The present invention provides compositions and methods for modulating the expression of mucin 1, transmembrane. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding mucin 1, transmembrane. Such compounds have been shown to modulate the expression of mucin 1, transmembrane.

BACKGROUND OF THE INVENTION

Mucins are high-molecular-weight, heavily glycosylated proteins found in milk, mammary gland and lactating tissue, as well as other simple secretory epithelial tissues. Mucins are constituents of the physical and biological barrier in protective mucous of respiratory, ductal and glandular epithelia. In humans, at least 10 distinct epithelial mucin core polypeptide genes have been identified (MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, and MUC9), and these mucins share the common features of bearing tandem repeat domains rich in proline, serine and threonine residues and forming O-glycans, with N-acetylgalactosamine linkages at hundreds of sites. Mucins are purported to be the most polymorphic of all biological macromolecules produced by eukaryotic organisms (even more so than immunoglobulin and T cell receptors). Mucin O-glycans serve as epitopes representing blood group and as related genetically polymorphic antigens (Irimura et al., *J. Biochem. (Tokyo)*, **1999**, 126, 975-985).

The highly-glycosylated mucin-type glycoproteins present in human urine and several normal and malignant tissues of epithelial origin are very antigenic, and in searches for epithelial and tumor-associated antigens, a large number of

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monoclonal antibodies have been produced which bind to the mucins. These antibodies have been used in cancer diagnosis and therapy, as well as to study the expression and variation of the PUM (peanut lectin binding urinary mucins) antigens and to

5 confirm that the PUM locus, a highly-polymorphic "minisatellite" region of the genome, encodes a mammary mucin (Karlsson et al., *Ann. Hum. Genet.*, **1983**, 47, 263-269; Swallow et al., *Nature*, **1987**, 328, 82-84). A full-length cDNA encoding mucin 1, transmembrane (also known as MUC1, episialin, epitectin,

10 polymorphic epithelial mucin, PEM, peanut-reactive urinary mucin, PUM, epithelial membrane antigen, EMA, PAS-0, NCRC11, H23 antigen, H23-ETA transmembrane antigen, DF3 antigen, and CD227) was deduced from overlapping clones isolated from a cDNA library constructed from the BT20 breast cancer cell line. The mucin 1,

15 transmembrane gene encodes a protein with three distinct regions: a signal peptide and degenerate tandem repeats at the N-terminus; the major portion of the protein comprising 60-base pair repeats which form a variable number tandem repeats (VNTR) region, length varying with the individual; and a C-terminus

20 comprising degenerate tandem repeats, a unique transmembrane sequence and a cytoplasmic tail (Gendler et al., *J. Biol. Chem.*, **1990**, 265, 15286-15293). This VNTR region is expressed, and it accounts for the polymorphism observed in both the mucin 1, transmembrane gene and its protein product.

25 Concurrently, a monospecific polyclonal antiserum against deglycosylated human pancreatic tumor mucin was used to clone a mucin cDNA from an expression library prepared from the HPAF pancreatic tumor cell line (Lan et al., *J. Biol. Chem.*, **1990**, 265, 15294-15299). This cDNA was found to be distinct from

30 intestinal mucin, but to be 99% homologous to the human breast mucin cDNA cloned by Gendler, et al., leading to the suggestion that, although the native forms of the pancreatic and breast mucin proteins are distinct in size and degree of glycosylation, factors other than its primary sequence determine these

35 characteristics, and the core protein (referred to as apomucin by Lan et al.) is encoded by same gene, hereafter referred to as mucin 1, transmembrane. Northern analyses of RNA from pancreatic and breast adenocarcinoma and colon tumor cell lines revealed a

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4.4-kilobase (kb) mucin 1, transmembrane mRNA in 5 of 7
pancreatic tumor cell lines and two of two breast tumor cell
lines, whereas no transcript was detected in the mucin-producing
colon tumor lines tested. In addition to the 4.4 kb transcript,
5 a larger mRNA with heterogeneous sizes greater than 7 kb was
observed in the Colo 357 pancreatic cell line (Lan et al., *J.*
Biol. Chem., **1990**, 265, 15294-15299).

A series of human-rodent somatic cell hybrids were used to
map the PUM locus to human chromosome 1, and by *in situ*
10 hybridization, the mucin 1, transmembrane gene was more finely
mapped to the 1q21-24 region (Swallow et al., *Ann. Hum. Genet.*,
1987, 51, 289-294). The gene coding for Duffy blood group FY is
closely linked to this same region (Swallow et al., *Ann. Hum.*
Genet., **1988**, 52, 269-271) and close linkage of mucin 1,
15 transmembrane to alpha-spectrin, a major component of the
erythrocyte membrane, confirms the position of mucin 1,
transmembrane at chromosomal locus 1q21 (Middleton-Price et al.,
Ann. Hum. Genet., **1988**, 52, 273-278).

The extracellular variable tandem repeat domain of mucin 1,
20 transmembrane protein is highly O-glycosylated, with each 20
amino acid repeat bearing five potential glycosylation sites.
Aberrant glycosylation has been described in malignancies. Due
to the VNTRs, aberrant glycosylation, and alternative splicing,
a considerable number of mucin 1, transmembrane isoforms have
25 been described. To date, these are: MUC1, the so-called "normal"
isoform; MUC1/REP, expressed in cervical cancer; MUC1/A, the
"cancer-specific" isoform found in thyroid carcinoma tissue;
MUC1/SEC, lacking the transmembrane domain and is a secreted
isoform; MUC1/X, MUC1/Y, and MUC1/Z which lack the VNTR region;
30 and two recently identified splice variants, MUC1/C, MUC1/D,
expressed in cervical carcinoma (Obermair et al., *Gynecol.*
Oncol., **2001**, 83, 343-347).

In contrast to other mucins such as those secreted by
goblet cells of the inner lining of the intestine, airway, and
35 reproductive tract, mucin 1, transmembrane is an integral plasma
membrane protein localized to the apical surface of polarized
epithelial cells, including, but not limited to, the uterus,
cervix, and vagina, as well as secretory epithelial cells of the

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mammary gland (Mather et al., *Cell Tissue Res.*, **2001**, 304, 91-101), and to both normal and malignant lung epithelial cells (Griffiths et al., *Dis. Markers*, **1988**, 6, 195-202).

5 The cytoplasmic tail of mucin 1, transmembrane protein is believed to interact with actin filaments of the cytoskeleton, and its relatively large, highly glycosylated extracellular domain may present a physical barrier that protects the cell with anti-invasion characteristics. Mucin 1, transmembrane may help to frustrate infection in the mammary gland (mastitis) and
10 possibly in other sites in the body (such as bladder and kidney infections) by competitively inhibiting the binding of microorganisms. A mucin 1, transmembrane null mouse has been generated, and these knockout mice are predisposed to bacterial conjunctivitis and blepharitis, demonstrating an important role
15 for mucin 1, transmembrane in ocular mucosal defense (Kardon et al., *Invest. Ophthalmol. Vis. Sci.*, **1999**, 40, 1328-1335).

Mucin 1, transmembrane may also play a role the immune response, intracellular signaling, and in suppression of cell adhesion or wall-to-wall adherence in lumens and ducts,
20 preventing their closure and preserving the integrity of secretory systems. Tumor cells tend to express mucin 1, transmembrane aberrantly in a non-polarized manner, potentially facilitating their tumor invasion and metastasis to other locations, and consequently, mucin 1, transmembrane may be
25 associated with biologically aggressive tumors and a worse prognosis (Patton et al., *Biochim. Biophys. Acta*, **1995**, 1241, 407-423; Rahn et al., *Cancer*, **2001**, 91, 1973-1982).

The multiple functions of mucin 1, transmembrane in carcinoma-host interactions are believed to be dependent on its
30 polymorphic nature, particularly its glycosylation status. Many carcinoma-associated markers are glycoproteins whose expression undergoes temporal or spatial regulation, and mucin 1, transmembrane is such a molecule (Rahn et al., *Cancer*, **2001**, 91, 1973-1982). Several data suggest that mucin 1, transmembrane
35 plays a role in tumor progression and metastasis: an underglycosylated form of mucin 1, transmembrane is overexpressed in virtually all invasive breast carcinomas; mucin 1, transmembrane is overexpressed in advanced stage tumors and

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metastatic foci from colon carcinoma; and mucin 1, transmembrane overexpression is inversely correlated with post-surgical survival of renal cell carcinoma patients (Irimura et al., *J. Biochem. (Tokyo)*, **1999**, 126, 975-985). Expression of mucin 1, transmembrane is up-regulated in ovarian cancer cell lines (Hough et al., *Cancer Res.*, **2000**, 60, 6281-6287) and lung adenocarcinoma cell lines (Yu et al., *Oncology*, **1996**, 53, 118-126). Thus, mucin 1, transmembrane is a prime candidate for therapeutic strategies targeting this carcinoma associated antigen.

Mucin 1, transmembrane has been used as an immunotherapeutic target to elicit both humoral and cellular immunity. A double transgenic mouse model for pancreatic cancer that overexpresses large amounts of underglycosylated mucin 1, transmembrane protein and spontaneously develops mucin 1, transmembrane-expressing tumors of the pancreas has been used to study the native immune response. These mice raised low-affinity cytotoxic T-lymphocytes (CTLs) specific for mucin 1, transmembrane, and these CTLs can be stimulated to kill mucin 1, transmembrane-expressing cancer cell lines *in vitro*, and eradicate injectable tumors upon adoptive transfer (Mukherjee et al., *J. Immunol.*, **2000**, 165, 3451-3460). Similarly, vaccination of mice with a liposomal formulation that incorporates synthetic mucin 1, transmembrane-based lipopeptide and Lipid A into a 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/cholesterol bilayer resulted in production of interferon-gamma and a peptide-specific immunological response dependent on cholesterol content (Batenjany et al., *Biochim. Biophys. Acta*, **2001**, 1514, 280-290). In contrast to the response observed upon immunization of mice, cynomolgus monkeys immunized with a peptide fusion of 5 VNTRs of macaque mucin 1, transmembrane conjugated with oxidized mannan mounted a humoral immune response, but not a CTL autoimmune response (Vaughan et al., *Vaccine*, **2000**, 18, 3297-3309).

In human cells, the MA5 monoclonal antibody against mucin 1, transmembrane protein was used to explore the potential of mucin 1, transmembrane to serve as an antigenic target for radioimmunotherapy (RAIT). From these studies, it was concluded

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that radiolabelled MA5 demonstrated therapeutic potential in a majority of the multiple myeloma (MM) cells tested (Burton et al., *Clin. Cancer Res.*, **1999**, 5, 3065s-3072s).

5 A vector expressing the mucin 1, transmembrane cDNA in the antisense orientation was used to transfect the human pancreatic tumor cell line, Panc 1, (Batra et al., *J. Cell Sci.*, **1991**, 100, 841-849) or the carcinogen-induced hamster pancreatic ductal tumor cell line, HP-1 (Batra et al., *Int. J. Pancreatol.*, **1992**, 12, 271-283), and produce transgenic pancreatic cell lines.

10 Northern and western blot analyses demonstrated mucin 1, transmembrane mRNA and protein expression in cells transfected with the cDNA in the correct orientation with respect to the promoter, but not in control cells (HP-1 cells transfected with vector alone, or with the mucin 1, transmembrane cDNA in the

15 antisense orientation). Ultrastructural analyses of the mucin 1, transmembrane expressing transgenic human Panc 1 cells demonstrated the formation of dense core granules and increased amounts of rough endoplasmic reticulum, representing

20 morphological evidence of potentially increased secretory activity and cellular differentiation (Batra et al., *J. Cell Sci.*, **1991**, 100, 841-849). The integration of human mucin 1, transmembrane in hamster HP-1 cells caused no significant change in the growth rate of HP-1 cells in vitro, but resulted in an enhanced growth rate for xenografts of mucin 1, transmembrane

25 transfected HP-1 cells grown in nude mice (Batra et al., *Int. J. Pancreatol.*, **1992**, 12, 271-283).

An antisense oligonucleotide, 21 nucleotides in length, corresponding to a portion of the tandemly repeated sequence was used to as a control in an experiment testing the effect of MUC2

30 mucin antisense oligonucleotides on the expression of MUC2-related antigens. The effect of this antisense oligonucleotide on mucin 1, transmembrane gene expression was not assessed (Bergeron et al., *J. Biol. Chem.*, **1996**, 271, 6933-6940).

A phosphorothioate antisense oligonucleotide, of

35 unspecified sequence and length, was purchased from Biognosik GmbH (Göttingen, Germany) and used to inhibit expression of mucin 1, transmembrane, resulting in induction of E-cadherin-

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mediated cell adhesion in the YMB-S breast cancer cell line (Kondo et al., *Cancer Res.*, **1998**, 58, 2014-2019).

Disclosed and claimed in US Patents 5,861,381 and 6,203,795 are a pharmaceutical composition which comprises, as therapeutic agent, the polypeptide recognized by antibody H23 (which recognizes the mucin 1, transmembrane protein) as well as a vaccinia virus into the genome of which a DNA fragment coding for said polypeptide is inserted, said DNA fragment being placed under the control of suitable transcription and translation signals, said polypeptide comprising a sequence repeated n times, n being a number from 1 to 80. Further claimed is a method of treating or preventing a malignancy characterized by malignant tumors that express elevated amounts of the antigen recognized by the H23 antibody comprising administering a therapeutically or prophylactically effective amount of said pharmaceutical composition (Chambon et al., **2001**; Chambon et al., **1999**).

Disclosed and claimed in European Patent EP1103623 is a nucleic acid fragment comprising at least 17 nucleotide bases the fragment being hybridizable with at least one of a group of sequences representing the tandemly-repeated sequences within mucin 1, transmembrane. Also claimed is a nucleic acid fragment comprising a portion of at least 30 nucleotide bases capable of hybridizing with at least one of said tandemly-repeated sequences, a double stranded DNA fragment comprising antiparallel paired portions having said sequences, said nucleic acid fragments for use in a method of therapy or diagnosis practiced on the human or animal body, an antibody or fragment thereof against a human mucin core protein which antibody or fragment has reduced or substantially no reaction with fully expressed human mucin glycoprotein, human polymorphic epithelial mucin core protein, a polypeptide comprising 5 or more amino acid residues in a sequence corresponding to a portion of mucin 1, transmembrane protein, and a diagnostic or therapeutic method practiced on the human or animal body comprising administering an antibody or fragment thereof, or human polymorphic epithelial mucin core protein (Taylor-Papadimitriou et al., **2001**).

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To date, investigative strategies aimed at modulating mucin 1, transmembrane function have involved the use of antisense expression vectors, antisense oligonucleotides, and antibodies. Currently, however, there are no known therapeutic agents which effectively inhibit the synthesis of mucin 1, transmembrane.

Consequently, there remains a long felt need for agents capable of effectively inhibiting mucin 1, transmembrane function.

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of mucin 1, transmembrane expression.

The present invention provides compositions and methods for modulating mucin 1, transmembrane expression, including modulation of variants of mucin 1, transmembrane.

SUMMARY OF THE INVENTION

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding mucin 1, transmembrane, and which modulate the expression of mucin 1, transmembrane. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of mucin 1, transmembrane in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of mucin 1, transmembrane by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating

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the function of nucleic acid molecules encoding mucin 1, transmembrane, ultimately modulating the amount of mucin 1, transmembrane produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding mucin 1, transmembrane. As used herein, the terms "target nucleic acid" and "nucleic acid encoding mucin 1, transmembrane" encompass DNA encoding mucin 1, transmembrane, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of mucin 1, transmembrane. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding mucin 1, transmembrane. The

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targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding mucin 1, transmembrane, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50

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contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also
5 a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides
10 between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the
15 translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap
20 structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as
25 "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are
30 particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can
35 also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA.

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These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and extronic regions. Upon excision of one or more exon or intron regions or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more than one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position

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of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The
5 oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a
10 sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be
15 specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific
20 binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

25 Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred
30 sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research reagents
35 and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also

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used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene expression) (Madden, et al., *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Jungblut, et al., *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Larsson, et al., *J. Biotechnol.*, **2000**, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, **2000**, 286, 91-98; Larson, et al., *Cytometry*, **2000**, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, **1998**, 31, 286-96), FISH (fluorescent in situ hybridization)

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techniques (Going and Gusterson, *Eur. J. Cancer*, **1999**, 35, 1895-904) and mass spectrometry methods (reviewed in (To, *Comb. Chem. High Throughput Screen*, **2000**, 3, 235-41).

The specificity and sensitivity of antisense is also
5 harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been
10 safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term
15 "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides
20 having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased
25 stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The
30 antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30
35 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its

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expression.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked

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analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307;

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5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240;
5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312;
5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439,
certain of which are commonly owned with this application, and
5 each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar
and the internucleoside linkage, i.e., the backbone, of the
nucleotide units are replaced with novel groups. The base units
are maintained for hybridization with an appropriate nucleic
10 acid target compound. One such oligomeric compound, an
oligonucleotide mimetic that has been shown to have excellent
hybridization properties, is referred to as a peptide nucleic
acid (PNA). In PNA compounds, the sugar-backbone of an
oligonucleotide is replaced with an amide containing backbone,
15 in particular an aminoethylglycine backbone. The nucleobases
are retained and are bound directly or indirectly to aza
nitrogen atoms of the amide portion of the backbone.

Representative United States patents that teach the preparation
of PNA compounds include, but are not limited to, U.S.:

20 5,539,082; 5,714,331; and 5,719,262, each of which is herein
incorporated by reference. Further teaching of PNA compounds
can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are
oligonucleotides with phosphorothioate backbones and
25 oligonucleosides with heteroatom backbones, and in particular -
CH₂-NH-O-CH₂-, -CH₂-N(CH₃)-O-CH₂- [known as a methylene
(methylinino) or MMI backbone], -CH₂-O-N(CH₃)-CH₂-, -CH₂-N(CH₃)-
N(CH₃)-CH₂- and -O-N(CH₃)-CH₂-CH₂- [wherein the native
phosphodiester backbone is represented as -O-P-O-CH₂-] of the
30 above referenced U.S. patent 5,489,677, and the amide backbones
of the above referenced U.S. patent 5,602,240. Also preferred
are oligonucleotides having morpholino backbone structures of
the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more
35 substituted sugar moieties. Preferred oligonucleotides comprise
one of the following at the 2' position: OH; F; O-, S-, or N-
alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-
alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted

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or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3 , OCN, Cl, Br, CN, CF_3 , OCF_3 , $SOCH_3$, SO_2CH_3 , ONO_2 , NO_2 , N_3 , NH_2 , heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O- $CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O- CH_2 -O- CH_2 -N(CH_3)₂, also described in examples hereinbelow.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene ($-CH_2-$)_n group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O- CH_3), 2'-aminopropoxy (2'-O- $CH_2CH_2CH_2NH_2$), 2'-allyl (2'- CH_2 -CH=CH₂), 2'-O-allyl (2'-O- CH_2 -CH=CH₂) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides

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may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoinidole cytidine (H-

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pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one

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or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers.

Typical conjugates groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides &*

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Nucleotides, **1995**, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxysterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, 277, 923-937. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethacin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense

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compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for

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such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In

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particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with

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organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylemaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from

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elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of mucin 1, transmembrane is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding mucin 1, transmembrane, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding mucin 1, transmembrane can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of mucin 1, transmembrane in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or

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intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C₁₋₁₀ alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents,

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emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred

5 surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic

10 acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, . Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid,

15 linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration

20 enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the

25 invention may be delivered orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins,

30 albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine,

35 polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-

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methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for
5 oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999) each of which is incorporated herein by reference
10 in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration
15 enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be
20 generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be
25 prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing
30 into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited
35 to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain

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substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active

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drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed.

5 Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not.

10 Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

15 Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the

20 emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic

25 surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents,

30 have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988,

35 volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has

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been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group:

5 nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

10 Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also
15 been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal
20 magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty
25 acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
30 and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum,
35 karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to

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form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and

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Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth
5 component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah,
10 *in: Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of
15 the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co.,
20 Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*,
25 Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of
30 solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol
35 fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750),

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decaglycerol sequioleate (S0750), decaglycerol decaoleate (DA0750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385; Ho et al., *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and

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pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

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In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is
5 highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their
10 internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the
15 aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge
20 with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is
25 growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to
30 administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and
35 high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

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Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, **1987**, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, **1992**, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, **1992**, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and

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concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, **1992**, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P. Pharma. Sci.*, **1994**, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, **1987**, 223, 42; Wu et al., *Cancer Research*, **1993**, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, **1987**, 507, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, **1988**, 85,

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6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, **1980**, 53, 2778) described liposomes comprising a nonionic detergent, 2C₁₂15G, that contains a PEG moiety. Illum et al. (*FEBS Lett.*, **1984**, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Lett.*, **1990**, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta*, **1990**, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.). Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.). U.S. Patent Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-

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containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses
5 methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of
10 encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive
15 candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.* they are self-
20 optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver
25 serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most
30 common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different
35 surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide

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application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

35 . Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of

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animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C₁₋₁₀ alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier*

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Systems, **1991**, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, **1992**, 44, 651-654).

5 Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, **1996**, pp. 934-935). Various natural
10 bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically
15 acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid
20 (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92;
25 Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Yamamoto et al., *J. Pharm. Exp. Ther.*, **1992**, 263, 25; Yamashita et al., *J. Pharm. Sci.*, **1990**,
30 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes
35 therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as

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most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, 618, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Buur et al., *J. Control Rel.*, **1990**, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, **1987**, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

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Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, **1995**, 5, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, **1996**, 6, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants

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(e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the

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compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp. 1206-1228, Berkow et al., eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially

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(e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide).

5 Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual*
10 *of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

15 In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense
20 compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and
25 responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient.
30 Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50} s found to be effective in in vitro and in vivo animal models. In
35 general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on

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measured residence times and concentrations of the drug in
bodily fluids or tissues. Following successful treatment, it
may be desirable to have the patient undergo maintenance therapy
to prevent the recurrence of the disease state, wherein the
5 oligonucleotide is administered in maintenance doses, ranging
from 0.01 ug to 100 g per kg of body weight, once or more daily,
to once every 20 years.

While the present invention has been described with
specificity in accordance with certain of its preferred
10 embodiments, the following examples serve only to illustrate the
invention and are not intended to limit the same.

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EXAMPLES**Example 1****5 Nucleoside Phosphoramidites for Oligonucleotide Synthesis
Deoxy and 2'-alkoxy amidites**

2'-Deoxy and 2'-methoxy beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial sources (e.g. ChemGenes, Needham MA or Glen Research, Inc. Sterling VA).
10 Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole
15 and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, 21, 3197-3203] using commercially available phosphoramidites (Glen
20 Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites**2'-Fluorodeoxyadenosine amidites**

2'-fluoro oligonucleotides were synthesized as described
25 previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material
30 and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP
35 and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

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2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-
5 beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine.
10 Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

15 Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to
20 obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to
25 give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are
30 prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available
35 through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas

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to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was
5 decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give
10 a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp
15 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L)
20 were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were
25 filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g)
30 prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

35 2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of

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dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase was dried over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl₃ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl₃. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

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3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85

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g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotrope with MeOH (200 mL). The residue was dissolved in CHCl_3 (700 mL) and extracted with saturated NaHCO_3 (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO_4 and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)-phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO_3 (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over MgSO_4 and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case

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of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O²-2'-anhydro-5-methyluridine

5 O²-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 10 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (R_f 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated 15 sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to 20 -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

25 **5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine**

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, 30 excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O²-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an 35 internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (R_f 0.67 for desired product and R_f 0.82 for ara-T side product, ethyl acetate) indicated about 70%

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conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P₂O₅ under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

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5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidooxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH₂Cl₂ (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH₂Cl₂ and the combined organic phase was washed with water, brine and dried over anhydrous Na₂SO₄. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium *p*-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH₂Cl₂). Aqueous NaHCO₃ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na₂SO₄, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO₃,

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(25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

2'-O-(dimethylaminoxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine

2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

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5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5 5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine
(1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the
residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was
added and dried over P₂O₅ under high vacuum overnight at 40°C.
Then the reaction mixture was dissolved in anhydrous
10 acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N¹,N¹-
tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The
reaction mixture was stirred at ambient temperature for 4 hrs
under inert atmosphere. The progress of the reaction was
monitored by TLC (hexane:ethyl acetate 1:1). The solvent was
15 evaporated, then the residue was dissolved in ethyl acetate
(70mL) and washed with 5% aqueous NaHCO₃ (40mL). Ethyl acetate
layer was dried over anhydrous Na₂SO₄ and concentrated. Residue
obtained was chromatographed (ethyl acetate as eluent) to get
5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-
20 [(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam
(1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

25 2'-(Aminooxyethoxy) nucleoside amidites [also known in the
art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as
described in the following paragraphs. Adenosine, cytidine and
thymidine nucleoside amidites are prepared similarly.

30 **N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-
5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-
N,N-diisopropylphosphoramidite]**

35 The 2'-O-aminooxyethyl guanosine analog may be obtained by
selective 2'-O-alkylation of diaminopurine riboside. Multigram
quantities of diaminopurine riboside may be purchased from
Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl)
diaminopurine riboside along with a minor amount of the 3'-O-
isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be
resolved and converted to 2'-O-(2-ethylacetyl)guanosine by

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treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-([2-phthalamidoxy]ethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH₂-O-CH₂-N(CH₂)₂, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O²-, 2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

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5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH₂Cl₂ (2x200 mL). The combined CH₂Cl₂ layers are washed with saturated NaHCO₃ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH₂Cl₂:Et₃N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH₂Cl₂ (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68

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sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides,

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as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide

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segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

**[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)]
Chimeric Phosphorothioate Oligonucleotides**

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)]
chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester

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internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl diisopropyl phosphoramidites were purchased from

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commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyl-diisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 5 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis,

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Ribonuclease protection assays, or RT-PCR.

T-24 cells:

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum ((Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

30

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville, MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville, MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

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HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville, MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville, MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

MCF7:

The human breast carcinoma cell line MCF-7 was obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells were routinely cultured in DMEM low glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

When cells reached 70% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 100 μ L OPTI-MEMTM-1 reduced-serum medium (Invitrogen Corporation, Carlsbad, CA) and then treated with 130 μ L of OPTI-MEMTM-1 containing 3.75 μ g/mL LIPOFECTINTM (Invitrogen Corporation, Carlsbad, CA) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control

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oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control

5 oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCCAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control

10 oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control

15 oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

20

Example 10**Analysis of oligonucleotide inhibition of mucin 1, transmembrane expression**

Antisense modulation of mucin 1, transmembrane expression

25 can be assayed in a variety of ways known in the art. For example, mucin 1, transmembrane mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time

30 quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. The preferred method of RNA analysis of the present invention is the use of total cellular RNA as described in other examples herein.

Methods of RNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993.

35 Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons,

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Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of mucin 1, transmembrane can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to mucin 1, transmembrane can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-

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4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 60 μ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM
5 vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times
10 with 200 μ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 μ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated
15 on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

20 **Example 12**

Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia, CA) following the manufacturer's recommended procedures. Briefly, for cells grown
25 on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 150 μ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 150 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The
30 samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 1 minute. 500 μ L of Buffer RW1 was added to each well of the RNEASY 96™ plate and incubated for 15 minutes and the vacuum was
35 again applied for 1 minute. An additional 500 μ L of Buffer RW1

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was added to each well of the RNEASY 96™ plate and the vacuum was applied for 2 minutes. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 90 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 3 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 170 µL water into each well, incubating 1 minute, and then applying the vacuum for 3 minutes.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13

Real-time Quantitative PCR Analysis of mucin 1, transmembrane mRNA Levels

Quantitation of mucin 1, transmembrane mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., FAM, obtained from either Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 5' end of the probe and a quencher dye (e.g.,

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TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from Invitrogen, Carlsbad, CA. RT-PCR reactions were carried out by adding 20 µL PCR cocktail

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(2.5x PCR buffer (-MgCl₂), 6.6 mM MgCl₂, 375 μ M each of dATP, dCTP, dGTP and dTTP, 375 nM each of forward primer and reverse primer, 125 nM of probe, 4 Units RNase inhibitor, 1.25 Units PLATINUM® Taq, 5 Units MuLV reverse transcriptase, and 2.5x ROX dye) to 96 well plates containing 30 μ L total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the PLATINUM® Taq, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, Analytical Biochemistry, 1998, 265, 368-374.

In this assay, 170 μ L of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:350 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 30 μ L purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human mucin 1, transmembrane were designed to hybridize to a human mucin 1, transmembrane sequence, using published sequence information (GenBank accession number NM_002456.1, incorporated herein as SEQ ID NO:3). For human mucin 1, transmembrane the PCR primers were: forward primer: TGACTCTGGCCTTCCGAGAA (SEQ ID NO: 4) reverse primer: GCTGCTTCCGTTTATACTGATTG (SEQ ID NO: 5) and the PCR probe was: FAM-TACCATCAATGTCCACGACGTGGAGACA-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human

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GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO:7)

reverse primer: GAAGATGGTGGTGGGATTTTC (SEQ ID NO:8) and the PCR

probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA 3' (SEQ ID NO: 9)

5 where JOE (PE-Applied Biosystems, Foster City, CA) is the
fluorescent reporter dye) and TAMRA (PE-Applied Biosystems,
Foster City, CA) is the quencher dye.

10 **Example 14**

Northern blot analysis of mucin 1, transmembrane mRNA levels

Eighteen hours after antisense treatment, cell monolayers
were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-
TEST "B" Inc., Friendswood, TX). Total RNA was prepared
15 following manufacturer's recommended protocols. Twenty
micrograms of total RNA was fractionated by electrophoresis
through 1.2% agarose gels containing 1.1% formaldehyde using a
MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was
transferred from the gel to HYBOND™-N+ nylon membranes (Amersham
20 Pharmacia Biotech, Piscataway, NJ) by overnight capillary
transfer using a Northern/Southern Transfer buffer system (TEL-
TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by
UV visualization. Membranes were fixed by UV cross-linking
using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La
25 Jolla, CA) and then probed using QUICKHYB™ hybridization
solution (Stratagene, La Jolla, CA) using manufacturer's
recommendations for stringent conditions.

To detect human mucin 1, transmembrane, a human mucin 1,
transmembrane specific probe was prepared by PCR using the
30 forward primer TGA CTCTGGCCTTCCGAGAA (SEQ ID NO: 4) and the
reverse primer GCTGCTTCCGTTTATACTGATTG (SEQ ID NO: 5). To
normalize for variations in loading and transfer efficiency
membranes were stripped and probed for human glyceraldehyde-3-
phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

35 Hybridized membranes were visualized and quantitated using
a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular
Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels

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in untreated controls.

Example 15

5 **Antisense inhibition of human mucin 1, transmembrane expression
by chimeric phosphorothioate oligonucleotides having 2'-MOE
wings and a deoxy gap**

In accordance with the present invention, a series of
oligonucleotides were designed to target different regions of
10 the human mucin 1, transmembrane RNA, using published sequences
(GenBank accession number NM_002456.1, representing the main
mRNA of mucin 1, transmembrane, incorporated herein as SEQ ID
NO: 3; GenBank accession number AF125525.1, representing the
variant MUC1/Y, incorporated herein as SEQ ID NO: 10; GenBank
15 accession number AF348143.1, representing a variant of mucin 1,
transmembrane herein designated MUC1-II, incorporated herein as
SEQ ID NO: 11; GenBank accession number AI834269.1, representing
a variant of mucin 1, transmembrane herein designated MUC1-III,
the complement of which is incorporated herein as SEQ ID NO: 12;
20 GenBank accession number AW369441.1, representing a variant of
mucin 1, transmembrane herein designated MUC1-IV, incorporated
herein as SEQ ID NO: 14; GenBank accession number BG774910.1,
representing a variant of mucin 1, transmembrane herein
designated MUC1-V, incorporated herein as SEQ ID NO: 16; GenBank
25 accession number J05581.1, representing a variant of mucin 1,
transmembrane herein designated MUC1-VI, incorporated herein as
SEQ ID NO: 17; GenBank accession number M31823.1, representing a
variant of mucin 1, transmembrane herein designated MUC1-VII,
incorporated herein as SEQ ID NO: 18; GenBank accession number
30 M61170, representing a genomic sequence of mucin 1,
transmembrane, incorporated herein as SEQ ID NO: 19; GenBank
accession number U60259.1, representing the variant MUC1/X,
incorporated herein as SEQ ID NO: 20; and GenBank accession
number Z17325.1, representing the variant MUC1/D, incorporated
35 herein as SEQ ID NO: 21). The oligonucleotides are shown in
Table 1. "Target site" indicates the first (5'-most) nucleotide
number on the particular target sequence to which the
oligonucleotide binds. All compounds in Table 1 are chimeric

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oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human mucin 1, transmembrane mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of human mucin 1, transmembrane mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
199396	5'UTR	3	8	gaacagattcaagcagccag	0	22
199397	Start Codon	3	49	cccgggtgtcatggtggtggt	58	23
199398	Start Codon	3	52	gtgcccgggtgtcatggtggt	58	24
199399	Coding	3	65	gaaaggagactgggtgcccg	54	25
199400	Coding	3	105	ctgtaacaactgtaagcact	41	26
199401	Coding	3	107	acctgtaacaactgtaagca	53	27
199402	Coding	3	187	tcagtagagctgggcactga	55	28
199403	Coding	3	196	gcattcttctcagtagagct	77	29
199404	Coding	3	197	agcattcttctcagtagagc	50	30
199405	Coding	3	210	tggtcatactcacagcatte	42	31
199406	Coding	3	214	ctgctgggtcatactcacagc	56	32
199407	Coding	3	227	gctggagagtacgctgctgg	57	33
199408	Coding	3	344	tgggaccgaggtgacatcct	65	34
199409	Coding	3	694	gtgacattgtggactggagg	55	35
199410	Coding	3	697	gaggtgacattgtggactgg	57	36
199411	Coding	3	704	tgaggccgaggtgacattgt	54	37
199412	Coding	3	829	gtggtaggagtatcagagtg	53	38
199413	Coding	3	835	gcaaggggtggtaggagtatc	50	39
199414	Coding	3	860	ggcatcagtccttggtgctat	53	40
199415	Coding	3	940	gagaccccagtagacaactg	24	41
199416	Coding	3	997	tcttccagagaggaattaaa	41	42
199417	Coding	3	1037	aatgtctctctgcagctctt	41	43
199418	Coding	3	1042	tcagaaatgtctctctgcag	54	44
199419	Coding	3	1056	tctgcaaaaacatttcagaa	45	45

199420	Coding	3	1065	gtttataaatctgcaaaaac	39	46
199421	Coding	3	1091	attggagaggcccagaaaac	41	47
199422	Coding	3	1095	taatattggagaggcccaga	50	48
199423	Coding	3	1100	gaacttaatatattggagaggc	48	49
199424	Coding	3	1112	agatcctggcctgaacttaa	53	50
199425	Coding	3	1115	cacagatcctggcctgaact	49	51
199426	Coding	3	1168	acgtcgtggacattgatggt	84	52
199427	Coding	3	1217	gttatatcgagaggctgctt	50	53
199428	Coding	3	1225	atcgtcaggttatatcgaga	47	54
199429	Coding	3	1251	gcacatcactcacgctgacg	50	55
199430	Coding	3	1268	ggcagagaaaggaaatggca	46	56
199431	Coding	3	1371	gacagacagccaaggcaatg	47	57
199432	Coding	3	1397	ctgcccgtagttctttcggc	43	58
199433	Coding	3	1412	tggaaagatgtccagctgcc	41	59
199434	Coding	3	1499	gctacgatcggtagctgtag	52	60
199435	Coding	3	1540	aggctgctgccaccattacc	59	61
199436	Coding	3	1582	aagttggcagaagtggctgc	42	62
199437	Stop Codon	3	1586	ctacaagttggcagaagtgg	35	63
199438	Stop Codon	3	1594	acgtgcccctacaagttggc	57	64
199439	3'UTR	3	1606	gctcagagggcgacgtgcc	36	65
199440	3'UTR	3	1617	ctggccactcagctcagagg	56	66
199441	3'UTR	3	1622	actggctggccactcagctc	55	67
199442	3'UTR	3	1630	ggaatggcactggctggcca	60	68
199443	3'UTR	3	1635	ggagtggaaatggcactggct	56	69
199444	Coding	10	141	aggaattaaaagcattcttc	7	70
199445	Coding	11	174	cagtagacaaagcattcttc	40	71
199446	Coding	11	297	gacagacagccatttcagaa	80	72
199447	Exon: Exon Junction	12	49	catcactcactgaacttaat	1	73
199448	Intron 6	19	5327	tttgggttttccaagtaccc	83	74
199449	Intron 6	19	5436	catagtctcctcccaggcct	44	75
199450	Intron 6	19	5588	cattttgcctctgggtgcaa	49	76
199451	Exon: Exon Junction	14	160	cagccccagacatttcagaa	21	77
199452	Intron 1	19	3289	ttctctctgcccataaggcct	42	78
199453	Intron 1	19	3426	gggtctttatgaaggaaaaa	43	79
199454	Exon: Exon Junction	16	455	acatcactcacatttcagaa	62	80
199455	3'UTR	17	1776	accacgttttattcagtcca	65	81
199456	Coding	18	115	gctgtggttagctgtaagcac	38	82
199457	Coding	20	175	gtgctgggatagcattcttc	15	83
199458	Coding	20	245	agagtcaattgtaccaccac	2	84
199459	Coding	21	122	ttttctccacctgtaagcac	18	85
199460	Intron: Exon Junction	19	3489	cctgtaacaactgttgcggg	32	86
199461	Intron: Exon	19	3498	tgaccagaacctgtaacaac	38	87

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	Junction					
199462	Exon 2d	19	3530	tctcctttttctccacctggg	49	88
199463	Exon 2d	19	3571	ctcagtagagctgggcactg	47	89
199464	Exon 2d	19	3590	tcatactcacagcattcttc	42	90
199465	Exon: Intron Junction	19	3973	agagcctgaggccgaggtga	58	91
199466	Intron: Exon Junction	19	4201	gaccccagtagacaactggg	20	92
199467	Intron: Exon Junction	19	4250	aggaattaaactggaggttt	55	93
199468	Exon 3d	19	4269	gtgctgggatcttccagaga	61	94
199469	Intron: Exon Junction	19	4621	atcctggcctgggtcacagg	39	95
199470	Exon 5	19	4936	cagccccagactgggcagag	41	96
199471	Intron 6	19	5449	ggcccttttcttccatagtc	55	97
199472	Intron 6	19	5889	ccacctggagtgtttttcca	42	98
199473	Intron 6	19	5956	aaagccgagagagggaggtc	51	99

As shown in Table 1, SEQ ID NOs 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 42, 43, 44, 45, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 64, 66, 67, 68, 69, 72, 74, 75, 76, 78, 79, 80, 81, 88, 89, 90, 91, 93, 94, 96, 97, 98 and 99 demonstrated at least 41% inhibition of human mucin 1, transmembrane expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

15 **Example 16**

Western blot analysis of mucin 1, transmembrane protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to mucin 1, transmembrane is used,

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with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

5

Example 17**Targeting of individual oligonucleotides to specific variants of mucin 1, transmembrane**

It is advantageous to selectively inhibit the expression of one or more variants of mucin 1, transmembrane. Consequently, in one embodiment of the present invention are oligonucleotides that selectively target, hybridize to, and specifically inhibit one or more, but fewer than all of the variants of mucin 1, transmembrane. A summary of the target sites of the variants is shown in Table 2 and includes Genbank accession number NM_002456.1, representing mucin 1, transmembrane (MUC1), incorporated herein as SEQ ID NO: 3; Genbank accession number AF125525.1, representing MUC1/Y, incorporated herein as SEQ ID NO: 10; Genbank accession number AF348143.1, representing MUC1-II, incorporated herein as SEQ ID NO: 11; Genbank accession number AI834269.1, representing MUC1-III, incorporated herein as SEQ ID NO: 12; Genbank accession number AW369441.1, representing MUC1-IV, incorporated herein as SEQ ID NO: 14; Genbank accession number BG774910.1, representing MUC1-V, incorporated herein as SEQ ID NO: 16; Genbank accession number J05581.1, representing MUC1-VI, incorporated herein as SEQ ID NO: 17; Genbank accession number M31823.1, representing MUC1-VII, incorporated herein as SEQ ID NO: 18; Genbank accession number U60259.1, representing MUC1/X, incorporated herein as SEQ ID NO: 20; Genbank accession number Z17325.1, representing MUC1/D, incorporated herein as SEQ ID NO: 21; Genbank accession number S81781.1, representing the variant MUC1/A, incorporated herein as SEQ ID NO: 100; Genbank accession number M32738.1, representing the variant MUC1/REP, incorporated herein as SEQ ID NO: 101; Genbank accession number M35093.1, representing the variant MUC1/SEC, incorporated herein as SEQ ID NO: 102; Genbank accession number U60261.1, representing the variant MUC1/Z, incorporated herein as SEQ ID NO: 103; Genbank accession number Z17324.1, representing the

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variant MUC1/C, incorporated herein as SEQ ID NO: 104; Genbank accession number BF876382.1, representing a variant of mucin 1, transmembrane herein designated MUC1-VIII, incorporated herein as SEQ ID NO: 105; Genbank accession number BG541121.1, representing a variant of mucin 1, transmembrane herein designated MUC1-IX, incorporated herein as SEQ ID NO: 106; Genbank accession number AL046435.1, representing a variant of mucin 1, transmembrane herein designated MUC1-X, incorporated herein as SEQ ID NO: 107.

10

Table 2

Targeting of individual oligonucleotides to specific variants of mucin 1, transmembrane

ISIS #	OLIGO SEQ ID NO.	TARGET SITE	VARIANT	VARIANT SEQ ID NO.
199396	22	8	MUC1	3
199397	23	49	MUC1	3
199397	23	16	MUC1-II	11
199397	23	64	MUC1-VI	17
199397	23	58	MUC1-VII	18
199397	23	17	MUC1/X	20
199397	23	65	MUC1/D	21
199397	23	1	MUC1/A	100
199397	23	42	MUC1/REP	101
199397	23	776	MUC1/SEC	102
199397	23	17	MUC1/Z	103
199397	23	65	MUC1/C	104
199397	23	59	MUC1-IX	106
199398	24	52	MUC1	3
199398	24	19	MUC1-II	11
199398	24	67	MUC1-VI	17
199398	24	61	MUC1-VII	18
199398	24	20	MUC1/X	20
199398	24	68	MUC1/D	21
199398	24	4	MUC1/A	100
199398	24	45	MUC1/REP	101
199398	24	779	MUC1/SEC	102
199398	24	20	MUC1/Z	103
199398	24	68	MUC1/C	104
199398	24	62	MUC1-IX	106
199399	25	65	MUC1	3
199399	25	8	MUC1/Y	10
199399	25	32	MUC1-II	11
199399	25	80	MUC1-VI	17
199399	25	74	MUC1-VII	18
199399	25	33	MUC1/X	20

199399	25	81	MUC1/D	21
199399	25	17	MUC1/A	100
199399	25	58	MUC1/REP	101
199399	25	792	MUC1/SEC	102
199399	25	33	MUC1/Z	103
199399	25	81	MUC1/C	104
199399	25	75	MUC1-IX	106
199400	26	105	MUC1	3
199400	26	72	MUC1-II	11
199400	26	120	MUC1-VI	17
199400	26	73	MUC1/X	20
199400	26	73	MUC1/Z	103
199401	27	107	MUC1	3
199401	27	74	MUC1-II	11
199401	27	122	MUC1-VI	17
199401	27	75	MUC1/X	20
199401	27	75	MUC1/Z	103
199402	28	187	MUC1	3
199402	28	121	MUC1/Y	10
199402	28	154	MUC1-II	11
199402	28	202	MUC1-VI	17
199402	28	223	MUC1-VII	18
199402	28	155	MUC1/X	20
199402	28	166	MUC1/A	100
199402	28	207	MUC1/REP	101
199402	28	1413	MUC1/SEC	102
199402	28	155	MUC1/Z	103
199402	28	346	MUC1-VIII	105
199402	28	224	MUC1-IX	106
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What is claimed is:

5 1. A compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding mucin 1, transmembrane, wherein said compound specifically hybridizes with said nucleic acid molecule encoding mucin 1, transmembrane and inhibits the expression of mucin 1, transmembrane.

10 2. The compound of claim 1 which is an antisense oligonucleotide.

 3. The compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 42, 15 43, 44, 45, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 64, 66, 67, 68, 69, 72, 74, 75, 76, 78, 79, 80, 81, 88, 89, 90, 91, 93, 94, 96, 97, 98 or 99.

 4. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside 20 linkage.

 5. The compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.

 6. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

25 7. The compound of claim 6 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

 8. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

30 9. The compound of claim 8 wherein the modified nucleobase is a 5-methylcytosine.

 10. The compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

35 11. A compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding mucin 1, transmembrane.

 12. A composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

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13. The composition of claim 12 further comprising a colloidal dispersion system.

14. The composition of claim 12 wherein the compound is an antisense oligonucleotide.

5 15. A method of inhibiting the expression of mucin 1, transmembrane in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of mucin 1, transmembrane is inhibited.

10 16. A method of treating an animal having a disease or condition associated with mucin 1, transmembrane comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of mucin 1, transmembrane is inhibited.

15 17. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

18. The method of claim 16 wherein the disease or disorder is an inflammatory disorder.

20 19. The compound of claim 1 targeted to a nucleic acid molecule encoding mucin 1, transmembrane, wherein said compound specifically hybridizes with and differentially inhibits the expression of one of the variants of mucin 1, transmembrane relative to the remaining variants of of mucin 1, transmembrane.

25 20. The compound of claim 19 targeted to a nucleic acid molecule encoding of mucin 1, transmembrane, wherein said compound hybridizes with and specifically inhibits the expression of a variant of of mucin 1, transmembrane, wherein said variant is selected from the group consisting of MUC1, MUC1/Y, MUC1/X, MUC1/D, MUC1/A, MUC1/REP, MUC1/SEC, MUC1/Z, MUC1/C, MUC1-II, MUC1-III, MUC1-IV, MUC1-V, MUC1-VI, MUC1-VII, 30 MUC1-VIII, MUC1-IX and MUC1-X.

SEQUENCE LISTING

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 Susan J. Myers
 Isis Pharmaceuticals, Inc.

<120> ANTISENSE MODULATION OF MUCIN 1, TRANSMEMBRANE EXPRESSION

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2

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Ala	Pro	Asp	Thr	Arg	Pro	Pro	Pro	Gly	Ser	Thr	Ala	Pro	Ala	Ala	His		
				165				170					175				
ggt	gtc	acc	tcg	gcc	ccg	gac	acc	agg	ccg	gcc	ccg	ggc	tcc	acc	gcc	633	
Gly	Val	Thr	Ser	Ala	Pro	Asp	Thr	Arg	Pro	Ala	Pro	Gly	Ser	Thr	Ala		
			180					185					190				
ccc	cca	gcc	cat	ggt	gtc	acc	tcg	gcc	ccg	gac	aac	agg	ccc	gcc	ttg	681	
Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	Asn	Arg	Pro	Ala	Leu		
		195					200					205					
gcg	tcc	acc	gcc	cct	cca	gtc	cac	aat	gtc	acc	tcg	gcc	tca	ggc	tct	729	
Ala	Ser	Thr	Ala	Pro	Pro	Val	His	Asn	Val	Thr	Ser	Ala	Ser	Gly	Ser		
	210					215					220						
gca	tca	ggc	tca	gct	tct	act	ctg	gtg	cac	aac	ggc	acc	tct	gcc	agg	777	
Ala	Ser	Gly	Ser	Ala	Ser	Thr	Leu	Val	His	Asn	Gly	Thr	Ser	Ala	Arg		
225					230					235					240		

gct acc aca acc cca gcc agc aag agc act cca ttc tca att ccc agc	825
Ala Thr Thr Thr Pro Ala Ser Lys Ser Thr Pro Phe Ser Ile Pro Ser	
245 250 255	
cac cac tct gat act cct acc acc ctt gcc agc cat agc acc aag act	873
His His Ser Asp Thr Pro Thr Thr Leu Ala Ser His Ser Thr Lys Thr	
260 265 270	
gat gcc agt agc act cac cat agc acg gta cct cct ctc acc tcc tcc	921
Asp Ala Ser Ser Thr His His Ser Thr Val Pro Pro Leu Thr Ser Ser	
275 280 285	
aat cac agc act tct ccc cag ttg tct act ggg gtc tct ttc ttt ttc	969
Asn His Ser Thr Ser Pro Gln Leu Ser Thr Gly Val Ser Phe Phe Phe	
290 295 300	
ctg tct ttt cac att tca aac ctc cag ttt aat tcc tct ctg gaa gat	1017
Leu Ser Phe His Ile Ser Asn Leu Gln Phe Asn Ser Ser Leu Glu Asp	
305 310 315 320	
ccc agc acc gac tac tac caa gag ctg cag aga gac att tct gaa atg	1065
Pro Ser Thr Asp Tyr Tyr Gln Glu Leu Gln Arg Asp Ile Ser Glu Met	
325 330 335	
ttt ttg cag att tat aaa caa ggg ggt ttt ctg ggc ctc tcc aat att	1113
Phe Leu Gln Ile Tyr Lys Gln Gly Phe Leu Gly Leu Ser Asn Ile	
340 345 350	
aag ttc agg cca gga tct gtg gtg gta caa ttg act ctg gcc ttc cga	1161
Lys Phe Arg Pro Gly Ser Val Val Val Gln Leu Thr Leu Ala Phe Arg	
355 360 365	
gaa ggt acc atc aat gtc cac gac gtg gag aca cag ttc aat cag tat	1209
Glu Gly Thr Ile Asn Val His Asp Val Glu Thr Gln Phe Asn Gln Tyr	
370 375 380	
aaa acg gaa gca gcc tct cga tat aac ctg acg atc tca gac gtc agc	1257
Lys Thr Glu Ala Ala Ser Arg Tyr Asn Leu Thr Ile Ser Asp Val Ser	
385 390 395 400	
gtg agt gat gtg cca ttt cct ttc tct gcc cag tct ggg gct ggg gtg	1305
Val Ser Asp Val Pro Phe Pro Phe Ser Ala Gln Ser Gly Ala Gly Val	
405 410 415	
cca ggc tgg ggc atc gcg ctg ctg gtg ctg gtc tgt gtt ctg gtt gcg	1353
Pro Gly Trp Gly Ile Ala Leu Leu Val Leu Val Cys Val Leu Val Ala	
420 425 430	
ctg gcc att gtc tat ctc att gcc ttg gct gtc tgt cag tgc cgc cga	1401
Leu Ala Ile Val Tyr Leu Ile Ala Leu Ala Val Cys Gln Cys Arg Arg	
435 440 445	
aag aac tac ggg cag ctg gac atc ttt cca gcc cgg gat acc tac cat	1449
Lys Asn Tyr Gly Gln Leu Asp Ile Phe Pro Ala Arg Asp Thr Tyr His	
450 455 460	
cct atg agc gag tac ccc acc tac cac acc cat ggg cgc tat gtg ccc	1497
Pro Met Ser Glu Tyr Pro Thr Tyr His Thr His Gly Arg Tyr Val Pro	
465 470 475 480	

cct agc agt acc gat cgt agc ccc tat gag aag gtt tct gca ggt aat 1545
 Pro Ser Ser Thr Asp Arg Ser Pro Tyr Glu Lys Val Ser Ala Gly Asn
 485 490 495

ggt ggc agc agc ctc tct tac aca aac cca gca gtg gca gcc act tct 1593
 Gly Gly Ser Ser Leu Ser Tyr Thr Asn Pro Ala Val Ala Ala Thr Ser
 500 505 510

gcc aac ttg tag gggcacgtcg ccctctgagc tgagtggcca gccagtgcc 1645
 Ala Asn Leu
 515

ttccactcca ctcagggctc tctgggccag tcctcctggg agccccacc acaacacttc 1705
 ccaggcatgg aattcc 1721

<210> 4
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>

<223> PCR Primer

<400> 4 20
 tgactctggc cttccgagaa

<210> 5
 <211> 24
 <212> DNA
 <213> Artificial Sequence

<220>

<223> PCR Primer

<400> 5 24
 gctgcttccg ttttatactg attg

<210> 6
 <211> 28
 <212> DNA
 <213> Artificial Sequence

<220>

<223> PCR Probe

<400> 6 28
 taccatcaat gtccacgacg tggagaca

<210> 7
 <211> 19
 <212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 7

gaaggtgaag gtcggagtc

19

<210> 8

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 8

gaagatggtg atgggatttc

20

<210> 9

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Probe

<400> 9

caagcttccc gttctcagcc

20

<210> 10

<211> 759

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)...(759)

<400> 10

atg	aca	ccg	ggc	acc	cag	tct	cct	ttc	ttc	ctg	ctg	ctg	ctc	ctc	aca	48
Met	Thr	Pro	Gly	Thr	Gln	Ser	Pro	Phe	Phe	Leu	Leu	Leu	Leu	Leu	Thr	
1				5				10					15			

gtg	ctt	aca	ggc	tct	ggc	cat	gca	agc	tct	acc	cca	ggc	gga	gaa	aag	96
Val	Leu	Thr	Gly	Ser	Gly	His	Ala	Ser	Ser	Thr	Pro	Gly	Gly	Glu	Lys	
			20				25					30				

gag	act	tcg	gct	acc	cag	aga	agt	tca	gtg	ccc	agc	tct	act	gag	aag	144
Glu	Thr	Ser	Ala	Thr	Gln	Arg	Ser	Ser	Val	Pro	Ser	Ser	Thr	Glu	Lys	
			35				40					45				

aat gct ttt aat tcc tct ctg gaa gat ccc agc acc gac tac tac caa	192
Asn Ala Phe Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp Tyr Tyr Gln	
50 55 60	
gag ctg cag aga gac att tct gaa atg ttt ttg cag att tat aaa caa	240
Glu Leu Gln Arg Asp Ile Ser Glu Met Phe Leu Gln Ile Tyr Lys Gln	
65 70 75 80	
ggg ggt ttt ctg ggc ctc tcc aat att aag ttc agg cca gga tct gtg	288
Gly Gly Phe Leu Gly Leu Ser Asn Ile Lys Phe Arg Pro Gly Ser Val	
85 90 95	
gtg gta caa ttg act ctg gcc ttc cga gaa ggt acc atc aat gtc cac	336
Val Val Gln Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile Asn Val His	
100 105 110	
gac atg gag aca cag ttc aat cag tat aaa acg gaa gca gcc tct cga	384
Asp Met Glu Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala Ala Ser Arg	
115 120 125	
tat aac ctg acg atc tca gac gtc agc gtg agt gat gtg cca ttt cct	432
Tyr Asn Leu Thr Ile Ser Asp Val Ser Val Ser Asp Val Pro Phe Pro	
130 135 140	
ttc tct gcc cag tct ggg gct ggg gtg cca ggc tgg ggc atc gcg ctg	480
Phe Ser Ala Gln Ser Gly Ala Gly Val Pro Gly Trp Gly Ile Ala Leu	
145 150 155 160	
ctg gtg ctg gtc tgt gtt ctg gtt gcg ctg gcc att gtc tat ctc att	528
Leu Val Leu Val Cys Val Leu Val Ala Leu Ala Ile Val Tyr Leu Ile	
165 170 175	
gcc ttg gct gtc tgt cag tgc cgc cga aag aac tac ggg cag ctg gac	576
Ala Leu Ala Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly Gln Leu Asp	
180 185 190	
atc ttt cca gcc cgg gat acc tac cat cct atg agc gag tac ccc acc	624
Ile Phe Pro Ala Arg Asp Thr Tyr His Pro Met Ser Glu Tyr Pro Thr	
195 200 205	
tac cac acc cat ggg cgc tat gtg ccc cct agc agt acc gat cgt agc	672
Tyr His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr Asp Arg Ser	
210 215 220	
ccc tat gag aag gtt tct gca ggt aat ggt ggc agc agc ctc tct tac	720
Pro Tyr Glu Lys Val Ser Ala Gly Asn Gly Gly Ser Ser Leu Ser Tyr	
225 230 235 240	
aca aac cca gca gtg gca gcc act tct gcc aac ttg tag	759
Thr Asn Pro Ala Val Ala Ala Thr Ser Ala Asn Leu	
245 250	

<210> 11

<211> 543

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (25)...(531)

<400> 11

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ctccccaccc atttcaccac cacc atg aca ccg ggc acc cag tct cct ttc      51
                Met Thr Pro Gly Thr Gln Ser Pro Phe
                  1                5

ttc ctg ctg ctg ctc ctc aca gtg ctt aca gtt gtt aca ggt tct ggt      99
Phe Leu Leu Leu Leu Leu Thr Val Leu Thr Val Val Thr Gly Ser Gly
  10                15                20                25

cat gca agc tct acc cca ggt gga gaa aag gag act tcg gct acc cag      147
His Ala Ser Ser Thr Pro Gly Gly Glu Lys Glu Thr Ser Ala Thr Gln
                30                35                40

aga agt tca gtg ccc agc tct act gag aag aat gct ttg tct act ggg      195
Arg Ser Ser Val Pro Ser Ser Thr Glu Lys Asn Ala Leu Ser Thr Gly
                45                50                55

gtc tct ttc ttt ttc ctg tct ttt cac att tca aac ctc cag ttt aat      243
Val Ser Phe Phe Phe Leu Ser Phe His Ile Ser Asn Leu Gln Phe Asn
                60                65                70

tcc tct ctg gaa gat ccc agc acc gac tac tac caa gag ctg cag aga      291
Ser Ser Leu Glu Asp Pro Ser Thr Asp Tyr Tyr Gln Glu Leu Gln Arg
  75                80                85

gac att tct gaa atg gct gtc tgt cag tgc cgc cga aag aac tac ggg      339
Asp Ile Ser Glu Met Ala Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly
  90                95                100                105

ctg ctg gac atc ttt cca gcc cgg gat acc tac cat cct atg agc gag      387
Leu Leu Asp Ile Phe Pro Ala Arg Asp Thr Tyr His Pro Met Ser Glu
                110                115                120

tac ccc acc tac cac acc cat ggg cgc tat gtg ccc cct agc agt acc      435
Tyr Pro Thr Tyr His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr
                125                130                135

gat cgt agc ccc tat gag aag gtt tct gca ggt aat ggt ggc agc agc      483
Asp Arg Ser Pro Tyr Glu Lys Val Ser Ala Gly Asn Gly Gly Ser Ser
                140                145                150

ctc tct tac aca aac cca gca gtg gca gcc act tct gcc aac ttg tag      531
Leu Ser Tyr Thr Asn Pro Ala Val Ala Ala Thr Ser Ala Asn Leu
  155                160                165

gggcacgtcg cc      543

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<210> 12

<211> 122

<212> DNA

<213> Homo sapiens

<220>

<221> exon:exon junction

<222> (58)...(59)

<223> exon 4:exon 6

<400> 12

atgtttttgc	agatttataa	acaaggggggt	tttctggggcc	tctccaatat	taagttcagt	60
gagtgatgtg	ccatttcctt	tctctgcccc	gtctgggggt	ggggtgccag	gctggggcat	120
cg						122

<210> 13

<211> 000

<212> DNA

<213> Homo sapiens

<220>

<400> 13

000

<210> 14

<211> 577

<212> DNA

<213> Homo sapiens

<220>

<221> exon:exon junction

<222> (169)...(170)

<223> exon 3c:exon 6b

<400> 14

cgtgtcgcga	ctgctcacct	cctccaatca	cagcacttct	ccccagttgt	ctactgggggt	60
ctctttcttt	ttcctgtctt	ttcacatttc	aaacctccag	tttaattcct	ctctggaaga	120
tcccagcacc	gactactacc	aagagctgca	gagagacatt	tctgaaatgt	ctggggctgg	180
ggtgccaggc	tggggcatcg	cgctgctggg	gctgggtctgt	gttctgggtg	cgctggccat	240
tgtctatctc	attgccttgg	ctgtctgtca	gtgccgccga	aagaactacg	ggcagctgga	300
catctttcca	gcccgggata	cctaccatcc	tatgagcgag	tacccacact	accacaccca	360
tgggcgctat	gtgcccccta	gcagtaccga	tcgtagcccc	tatgagaagg	tttctgcagg	420
taatggtggc	agcagcctct	cttacacaaa	cccagcagtg	gcagccactt	cttgcaactt	480
gtaggggcac	gtcgcccgct	gagctgagta	gccagccagt	gccattccac	tccactcagg	540
ttcttcaggg	ccagagcccc	tgcaccctgt	ttggggct			577

<210> 15

<211> 000

<212> DNA

<213> Homo sapiens

<220>

<400> 15

000

<210> 16

<211> 981

<212> DNA

<213> Homo sapiens

<220>

<221> exon:exon junction

<222> (464)...(465)

<223> exon 3b:exon 4

<400> 16

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gggacaccag gccggccccc gggtccaccg cccccccagc ccatgggtgtc acctcggccc      60
cggacaacag gccgccttg gggtccaccg cccctccagt ccacaatgtc acctcggcct      120
caggctctgc atcaggctca gcttctactc tgggtgcacaa cagcacctct gccagggcta      180
ccacaacccc agccagcaag agcactccat tctcaattcc cagccaccac tctgatactc      240
ctaccaccct tgccagccat agcaccaaga ctgatgccag tagcactcac catagcacgg      300
tacctcctct cacctcctcc aatcacagca ctctcctcca gttgtctact ggggtctctt      360
tctttttcct gtctttttcac atttcaaacc tccagtttaa ttctctcttg gaagatccca      420
gcaccgacta ctaccaagag ctgcagagag acattttctga aatgtgagtg atgtgccatt      480
tcctttctct gccagtcctg gggctggggg gccaggctgg ggcacgcgcg tgctgggtgt      540
gggtctgtgt ctggttgccg tggccattgt ctatctcatt gccttggctg tctgtcagtg      600
ccgccgaaag aactacgggc agctggacat ctttccagcc cgggatacct accatcctat      660
gagcgagtac cccacctacc aaccatggg cgtatgtgc cccctagcag taccgatcgt      720
agcccctatg agacaggttt ctgcaggtaa tgggtggcagc agctctctta cacaaccag      780
cagtggcagc cacttctgcc aacttgtagg ggcacgttgc cgctgacctg agtggccagc      840
cagtgccatt ccacttccac tcagggttct tcaggggcca gagccctgca cctgttttg      900
cctggtgagc tggacttcaa ggtgggctgt cacagcctct tcaaaggccc acaattcttc      960
gacatcctca ggtgtggaag c                                     981

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<210> 17

<211> 1804

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (73)...(1500)

<400> 17

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cgctccacct ctcaagcagc cagcgcctgc ctgaatctgt tctgccccct cccaccccat      60
ttcaccacca cc atg aca ccg ggc acc cag tct cct ttc ttc ctg ctg ctg      111
          Met Thr Pro Gly Thr Gln Ser Pro Phe Phe Leu Leu Leu
              1             5             10

ctc ctc aca gtg ctt aca gtt gtt aca ggt tct ggt cat gca agc tct      159
Leu Leu Thr Val Leu Thr Val Val Thr Gly Ser Gly His Ala Ser Ser
          15             20             25

acc cca ggt gga gaa aag gag act tcg gct acc cag aga agt tca gtg      207
Thr Pro Gly Gly Glu Lys Glu Thr Ser Ala Thr Gln Arg Ser Ser Val
          30             35             40             45

ccc agc tct act gag aag aat gct gtg agt atg acc agc agc gta ctc      255
Pro Ser Ser Thr Glu Lys Asn Ala Val Ser Met Thr Ser Ser Val Leu
              50             55             60

tcc agc cac agc ccc ggt tca ggc tcc tcc acc act cag gga cag gat      303
Ser Ser His Ser Pro Gly Ser Gly Ser Ser Thr Thr Gln Gly Gln Asp
              65             70             75

```

gtc act ctg gcc ccg gcc acg gaa cca gct tca ggt tca gct gcc acc	351
Val Thr Leu Ala Pro Ala Thr Glu Pro Ala Ser Gly Ser Ala Ala Thr	
80 85 90	
tgg gga cag gat gtc acc tcg gtc cca gtc acc agg cca gcc ctg ggc	399
Trp Gly Gln Asp Val Thr Ser Val Pro Val Thr Arg Pro Ala Leu Gly	
95 100 105	
tcc acc acc ccg cca gcc cac gat gtc acc tca gcc ccg gac aac aag	447
Ser Thr Thr Pro Pro Ala His Asp Val Thr Ser Ala Pro Asp Asn Lys	
110 115 120 125	
cca gcc ccg ggc tcc acc gcc ccc cca gcc cac ggt gtc acc tcg gcc	495
Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala	
130 135 140	
ccg gac acc agg ccg gcc ccg ggc tcc acc gcc ccc cca gcc cat ggt	543
Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His Gly	
145 150 155	
gtc acc tcg gcc ccg gac aac agg ccc gcc ttg ggc tcc acc gcc cct	591
Val Thr Ser Ala Pro Asp Asn Arg Pro Ala Leu Gly Ser Thr Ala Pro	
160 165 170	
cca gtc cac aat gtc acc tcg gcc tca ggc tct gca tca ggc tca gct	639
Pro Val His Asn Val Thr Ser Ala Ser Gly Ser Ala Ser Gly Ser Ala	
175 180 185	
tct act ctg gtg cac aac ggc acc tct gcc agg gct acc aca acc cca	687
Ser Thr Leu Val His Asn Gly Thr Ser Ala Arg Ala Thr Thr Thr Pro	
190 195 200 205	
gcc agc aag agc act cca ttc tca att ccc agc cac cac tct gat act	735
Ala Ser Lys Ser Thr Pro Phe Ser Ile Pro Ser His His Ser Asp Thr	
210 215 220	
cct acc acc ctt gcc agc cat agc acc aag act gat gcc agt agc act	783
Pro Thr Thr Leu Ala Ser His Ser Thr Lys Thr Asp Ala Ser Ser Thr	
225 230 235	
cac cat agc acg gta cct cct ctc acc tcc tcc aat cac agc act tct	831
His His Ser Thr Val Pro Pro Leu Thr Ser Ser Asn His Ser Thr Ser	
240 245 250	
ccc cag ttg tct act ggg gtc tct ttc ttt ttc ctg tct ttt cac att	879
Pro Gln Leu Ser Thr Gly Val Ser Phe Phe Phe Leu Ser Phe His Ile	
255 260 265	
tca aac ctc cag ttt aat tcc tct ctg gaa gat ccc agc acc gac tac	927
Ser Asn Leu Gln Phe Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp Tyr	
270 275 280 285	
tac caa gag ctg cag aga gac att tct gaa atg ttt ttg cag att tat	975
Tyr Gln Glu Leu Gln Arg Asp Ile Ser Glu Met Phe Leu Gln Ile Tyr	
290 295 300	
aaa caa ggg ggt ttt ctg ggc ctc tcc aat att aag ttc agg cca gga	1023
Lys Gln Gly Gly Phe Leu Gly Leu Ser Asn Ile Lys Phe Arg Pro Gly	

11

305	310	315	
tct gtg gtg gta caa ttg act ctg gcc ttc cga gaa ggt acc atc aat			1071
Ser Val Val Val Gln Leu Thr	Leu Ala Phe Arg Glu Gly Thr Ile Asn		
320	325	330	
gtc cac gac gtg gag aca cag ttc aat cag tat aaa acg gaa gca gcc			1119
Val His Asp Val Glu Thr	Gln Phe Asn Gln Tyr Lys Thr Glu Ala Ala		
335	340	345	
tct cga tat aac ctg acg atc tca gac gtc agc gtg agt gat gtg cca			1167
Ser Arg Tyr Asn Leu Thr	Ile Ser Asp Val Ser Val Ser Asp Val Pro		
350	355	360	365
ttt cct ttc tct gcc cag tct ggg gct ggg gtg cca ggc tgg ggc atc			1215
Phe Pro Phe Ser	Ala Gln Ser Gly Ala Gly Val Pro Gly Trp Gly Ile		
370	375		380
gcg ctg ctg gtg ctg gtc tgt gtt ctg gtt gcg ctg gcc att gtc tat			1263
Ala Leu Leu Val Leu Val Cys Val Leu Val Ala Leu Ala Ile Val Tyr			
385	390		395
ctc att gcc ttg gct gtc tgt cag tgc cgc cga aag aac tac ggg cag			1311
Leu Ile Ala Leu Ala Val Cys	Gln Cys Arg Arg Lys Asn Tyr Gly Gln		
400	405	410	
ctg gac atc ttt cca gcc cgg gat acc tac cat cct atg agc gag tac			1359
Leu Asp Ile Phe Pro Ala Arg Asp Thr Tyr His Pro Met Ser Glu Tyr			
415	420	425	
ccc acc tac cac acc cat ggg cgc tat gtg ccc cct agc agt acc gat			1407
Pro Thr Tyr His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr Asp			
430	435	440	445
cgt agc ccc tat gag aag gtt tct gca ggt aat ggt ggc agc agc ctc			1455
Arg Ser Pro Tyr Glu Lys Val Ser Ala Gly Asn Gly Gly Ser Ser Leu			
450	455	460	
tct tac aca aac cca gca gtg gca gcc act tct gcc aac ttg tag			1500
Ser Tyr Thr Asn Pro Ala Val Ala Ala Thr Ser Ala Asn Leu			
465	470	475	
gggcacgtcg cccgctgagc tgagtggcca gccagtgcc ttccactcca ctcaggttct			1560
tcagggccag agcccctgca cctgttttg gctggtgagc tgggagttca ggtgggctgc			1620
tcacaccgtc cttcagaggc cccaccaatt tctcggacac ttctcagtgt gtggaagctc			1680
atgtgggccc ctgaggctca tgccctgggaa gtgttggtgtg gggggctccc aggaggactg			1740
gccagagag ccctgagata gcgggggatcc tgaactggac tgaataaaac gtgggtctccc			1800
actg			1804

<210> 18

<211> 572

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (67)...(572)

<400> 18
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accacc atg aca ccg ggc acc cag tct cct ttc ttc ctg ctg ctg ctc 108
Met Thr Pro Gly Thr Gln Ser Pro Phe Phe Leu Leu Leu Leu
1 5 10

ctc aca gtg ctt aca gct acc aca gcc cct aaa ccc gca aca gtt gtt 156
Leu Thr Val Leu Thr Ala Thr Thr Ala Pro Lys Pro Ala Thr Val Val
15 20 25 30

acg ggt tct ggt cat gca agc tct acc cca ggt gga gaa aag gag act 204
Thr Gly Ser Gly His Ala Ser Ser Thr Pro Gly Gly Glu Lys Glu Thr
35 40 45

tcg gct acc cag aga agt tca gtg ccc agc tct act gag aag aat gct 252
Ser Ala Thr Gln Arg Ser Ser Val Pro Ser Ser Thr Glu Lys Asn Ala
50 55 60

gtg agt atg acc agc agc gta ctc tcc agc cac agc ccc ggt tca ggc 300
Val Ser Met Thr Ser Ser Val Leu Ser Ser His Ser Pro Gly Ser Gly
65 70 75

tcc tcc acc act cag gga cag gat gtc act ctg gcc ccg gcc acg gaa 348
Ser Ser Thr Thr Gln Gly Gln Asp Val Thr Leu Ala Pro Ala Thr Glu
80 85 90

cca gct tca ggt tca gct gcc acc tgg gga cag gat gtc acc tcg gtc 396
Pro Ala Ser Gly Ser Ala Ala Thr Trp Gly Gln Asp Val Thr Ser Val
95 100 105 110

cca gtc acc agg cca gcc ctg ggc tcc acc acc ccg cca gcc cac gat 444
Pro Val Thr Arg Pro Ala Leu Gly Ser Thr Thr Pro Pro Ala His Asp
115 120 125

gtc acc tca gcc ccg gac aac aag cca gcc ccg ggc tcc acc gcc ccc 492
Val Thr Ser Ala Pro Asp Asn Lys Pro Ala Pro Gly Ser Thr Ala Pro
130 135 140

caa gcc cac ggt gtc acc tcg gcc ccg gac acc agg ccg gcc ccg ggc 540
Gln Ala His Gly Val Thr Ser Ala Pro Asp Thr Arg Pro Ala Pro Gly
145 150 155

tcc acc gcc ccc caa gcc cac ggt gtc acc tc 572
Ser Thr Ala Pro Gln Ala His Gly Val Thr
160 165

<210> 19

<211> 8186

<212> DNA

<213> Homo sapiens

<220>

<221> unsure

<222> 6899

<223> unknown

<221> unsure

<222> 7155
<223> unknown

<221> unsure
<222> 7184
<223> unknown

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Phe Leu Leu Leu Leu Leu Thr Val Leu Thr Val Val Thr Gly Ser Gly
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```

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His Ala Ser Ser Thr Pro Gly Gly Glu Lys Glu Thr Ser Ala Thr Gln
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```

```

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Arg Ser Ser Val Pro Ser Ser Thr Glu Lys Asn Ala Ile Pro Ala Pro
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act act acc aag agc tgc aga gag aca ttt ctg aaa tgg cca gga tct 244
Thr Thr Thr Lys Ser Cys Arg Glu Thr Phe Leu Lys Trp Pro Gly Ser
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Val Val Val Gln Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile Asn Val
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His Asp Val Glu Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala Ala Ser
90                               95                               100                               105

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Arg Tyr Asn Leu Thr Ile Ser Asp Val Ser Val Ser Asp Val Pro Phe
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Pro Phe Ser Ala Gln Ser Gly Ala Gly Val Pro Gly Trp Gly Ile Ala
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Ile Ala Leu Ala Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly Gln Leu	
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Thr Tyr His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr Asp Arg	
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Ser Pro Tyr Glu Lys Val Ser Ala Gly Asn Gly Gly Ser Ser Leu Ser	
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Met Thr Pro Gly Thr Gln Ser Pro Phe Phe Leu Leu	
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<213> Homo sapiens

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<400> 105

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gcagagagac atttctgaaa tgtttttgca gatttataaa caagggggtt tcttgggcct 360
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ggacagaaca cgggccgctg gacctctttc ccgcccgga tacctacatc ctttgagggg 720
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<223> n = A,T,C or G

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gctggacatc tttccagccc gggataccta ccacacctatg agcgagtacc ccacctacca 180
caccatggg cgctatgtgc cccctagcag taccgatcgt agcccctatg agaaggtgag 240
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agcccttccc gaggagtcca aggggtgagc ttttg 635

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- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

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(54) Title: ANTISENSE MODULATION OF MUCIN 1, TRANSMEMBRANE EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of mucin 1, transmembrane. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding mucin 1, transmembrane. Methods of using these compounds for modulation of mucin 1, transmembrane expression and for treatment of diseases associated with expression of mucin 1, transmembrane are provided.



WO 03/054154 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/39873

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/02, 21/04; C12P 19/34; A61K 48/00; C12Q 1/68; C12N 15/85, 15/86;
US CL : 435/6, 91.1, 325, 375; 536/24.3, 24.31, 24.33, 24.5, 23.2, 23.1; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.1, 325, 375; 536/24.3, 24.31, 24.33, 24.5, 23.2, 23.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN, medline, caplus, lifesci, embase, uspatfull, biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENDLER ET AL. Molecular Cloning and Expression of Human Tumor-associated Polymorphic Epithelial Mucin. The Journal of Biological Chemistry. 05 September 1990, Vol. 265, No. 25, pages 15286-15293, see especially page 15287, first column, line 30.	1, 2, 11, 12, 14, 19, 20
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Y		4-10, 13, 15
X	BERGERON ET AL. MAUB Is a New Mucin Antigen Associated with Bladder Cancer. The Journal of Biological Chemistry. 22 March 1996, Vol. 271, No. 12, pages 6933-6940, see especially page 6935, first column, sixth paragraph, second column.	1, 2, 11, 12, 14, 15, 19, 20
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Y		4-10, 13
X	WO 00/34468 (BIOMIRA INC.) 15 June 2000 (15.06.00), see especially page 41, table IV, page 23, second paragraph.	1, 2, 4, 5, 11-15, 19, 20
---		-----
Y		6-10
Y	US 5,801,154 (BARACCHINI ET AL.) 01 September 1998 (01.09.98) see especially Columns 6-9.	1-15, 19, 20

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/39873

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claim Nos.: 3
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 3 is directed to nucleotide sequences, however, a Computer Readable Format of the sequence listing was not provided
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.